Olive oil phenolics prevent oxysterol-induced pro-inflammatory cytokine secretion and ROS production in human PBMCs, through modulation of p38 and JNK pathways.

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List of abbreviations: 25-HC, 25-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; 5α,6α-EC, cholesterol 5α,6α-epoxide; 5β,6β-EC, cholesterol 5β,6β-epoxide; 7-KC, 7-ketocholesterol; 7α-HC, 7α-hydroxycholesterol; 7β-HC, 7β-hydroxycholesterol; COX-2, cyclooxygenase-2; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DSS, dextran sulphate sodium; EVOO, extra virgin olive oil; HT, hydroxytyrosol; HVA, homovanillic alcohol; ICAM-1, cellular adhesion molecule-1; iNOS, inducible nitric oxide synthase; MAPK, mitogen activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MIF, macrophage migration inhibitory factor; MIP-1β, macrophage inflammatory protein-1β; NF-*k*B, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; PBMCs, peripheral blood mononuclear cells; RANTES, regulated on activation, normal T cell expressed and secreted; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; triol, cholestane-3β,5α,6β-triol; TYR, tyrosol; VCAM-1, vascular cell adhesion molecule-1.

Abstract

Scope: The aim of the present study was to investigate the ability of extra virgin olive oil (EVOO) polyphenols to counteract the pro-inflammatory effects induced by dietary and endogenous oxysterols in *ex-vivo* immune cells.

Methods and results: Peripheral blood mononuclear cells (PBMCs), separated from the whole blood of healthy donors, were utilised and were stimulated with an oxysterols mixture, in the presence of physiologically relevant concentrations of the EVOO polyphenols, hydroxytyrosol (HT), tyrosol (TYR) and homovanillic alcohol (HVA).

Oxysterols significantly increased the production of pro-inflammatory cytokines, interleukin-1β (IL-1β), regulated on activation, normal T cell expressed and secreted (RANTES) and macrophage migration inhibitory factor (MIF) in *ex-vivo* cultured PBMCs. Increased levels of reactive oxygen species (ROS) were also detected along with increased phosphorylation of the p38 and JNK. All phenolic compounds significantly reduced cytokine secretion induced by the oxysterols and inhibited ROS production and MAPK phosphorylation.

Conclusions: These results suggest that extra virgin olive oil polyphenols modulate the immune response induced by dietary and endogenous cholesterol oxidation products in human immune cells and may hold benefit in controlling chronic immune and/or inflammatory processes.

1. Introduction

Oxysterols are 27-carbon-atom molecules resulting from non-enzymatic or enzymatic oxidation of cholesterol and have been detected in plasma and tissues due to endogenous formation and dietary intake [1-3]. High levels of oxysterols, generated by dysfunction in endogenous production or through high dietary intake, can affect cellular metabolism, change membrane composition/property, and promote the onset and progression of major chronic and degenerative diseases such as cancer and atherosclerosis [4, 5]. It is thought that such deleterious effects are due to their ability to trigger cytotoxic, pro-oxidative and pro-inflammatory reactions [6], such as the production of superoxide anions (O^{-2}) and reactive oxygen species (ROS), or through their potential to enhance pro-inflammatory cytokine expression and secretion levels (tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-8, monocyte inflammatory protein-1 (MIP-1β), cellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin [7]. Notably, the expression of these and other inflammatory mediators is closely dependent on the activity nuclear factor kappa-light-chain-enhancer of activated B cells (NF-*K*b), through activation of the mitogen activated protein kinase (MAPK) pathways [8].

Extra virgin olive oil, the main fat source in the Mediterranean diet, with its high content in monounsaturated fatty acids and relatively high polyphenol content, may attenuate such inflammatory responses and exert beneficial effects in modulating chronic low-grade inflammation [9-13]. EVOO and its bioactive minor components, in particular, oleuropein [14, 15], hydroxytyrosol [16-21] and oleocanthal [22, 23], but also the entire phenolic fraction [24, 25], have been largely investigated in *in vitro* models and many findings support their anti-inflammatory and immune-modulatory effects.

This is thought to occur through their capacity to limit the ROS and nitrogen species formation [16, 24-28] and to inhibit the pro-inflammatory activity of ROS-generating enzymes including cyclooxygenase-2 (COX-2) [18, 24, 25, 27, 28], inducible nitric oxide synthase (iNOS) [18, 20, 24,

25, 28, 29] and to modulate different intracellular signalling pathways from NF-*k*B to MAPK through perturbation of redox-sensible networks in immune cells [26].

Also many *in vivo* studies have suggested that EVOO with high phenolic concentration is effective in modulating inflammatory mediator derived from arachidonic acid, such as TBX_2 and LTB_4 [30-33] as well as other inflammatory markers, such as high-sensitivity C-reactive protein [13, 34], IL-6 [10, 34, 35], IL-β [36], IFN-λ and IL-7 [30, 37],VCAM-1, ICAM-1, TNF-α and Monocyte Chemoattractant Protein-1 (MCP-1) [10].

The aim of this study was to investigate the ability of different olive oil pure phenolic compounds, (HT, TYR and HVA), found in the blood after ingestion and absorption of EVOO, to prevent inflammatory effects induced by oxysterols in human immune cells, and to understand the implicated mechanism of action.

PBMCs, separated from whole blood of healthy donors, were used for this purpose; these cells are a good model to study the inflammatory responses *ex-vivo* since they are composed by lymphocytes, monocytes and macrophages, critical components in the immune system to fight inflammation and source of pro-inflammatory molecules.

PBMCs were treated with an oxysterols mixture composed by the most widely represented oxysterols in plasma of hypercholesterolemic subjects: 7α-hydroxycholesterol (7α-HC), 7β-hydroxycholesterol (7β-HC), 7-ketocholesterol (7-KC), cholesterol 5α,6α-epoxide (5α,6α-EC), cholesterol 5β,6β-epoxide (5β,6β-EC), cholestane-3β,5α,6β-triol (triol) and 25-hydroxycholesterol (25-HC) [38] at pathologically relevant concentration (20 μM). PBMCs were used in order to investigate the ability of the olive oil simple phenols to inhibit the increase of ROS and pro-inflammatory cytokine/chemokine synthesis (IL-1β, MIF and RANTES) induced by the oxysterols mixture, and to modulate the signaling pathways (MAPK) involved in these processes.

2. Materials and Methods

2.1 Reagents

Media and supplements were purchased from Lonza (Slough, UK). Histopaque-1077, oxysterols, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, Bradford reagent, and solvents were purchased from Sigma Aldrich (Poole, UK). The cytokine kits were purchased from R&D systems (Abingdon, UK). Gels and all material for electrophoresis and immunoblotting were obtained from Invitrogen (Milan, Italy). The Western Blotting System was from Bio-Rad (Milan, Italy). The primary antibodies were purchased from Millipore (Watford, UK).

2.2 Isolation and culture of human peripheral blood mononuclear cells

The study was approved by the Ethics and Research Committee of the University of Reading (Project No. 12/16) and informed consent obtained from each blood donor.

The volunteers (males and females, aged between 23 and 40 years) were enrolled according to specific inclusion (signed informed consent, men and women aged 20 – 40 years, non smokers, good general health) and exclusion criteria (history of drug abuse, including alcohol, participation in experimental trials within 3 months prior to study, use of antibiotics within the previous 3 months, use of prescribed medication, regular use of anti-inflammatory drugs, any kind of inflammatory, auto-immune disease or allergy, any other pathology).

Overnight fasting (12 hours) venous blood samples from 14 healthy donors were collected in the morning in sodium-heparin coated tubes (Greiner Bio-One Limited, Gloucestershire, UK). PBMCs were immediately isolated by a Ficoll-Hypaque (Histopaque-1077) density gradient from 5 ml of whole peripheral blood following the manufacturer instructions and re-suspended in the culture medium consisting of RPMI 1640, 1% v/v glutamine and 1% v/v antibiotics, supplemented with autologous plasma (2.5% v/v). PBMCs were counted and cultured at 37°C in a 5% CO_2 humidified atmosphere.

2.3 Cell treatments for cytokines analysis

PBMCs ($1x10^6$ cells/ml) were seeded in 24 well plate in complete RPMI, pre-treated or not with HT, TYR and HVA (0.25, 0.5, 1 µM) and incubated for 30 min at 37°C in a 5% CO_2 atmosphere. Then, the oxysterols mixture 20 µM in ethanol was added in the medium for 24 h.

An equivalent amount of ethanol was added to the control cells for all the treatments.

At the end of the incubation period well contents were removed, transferred to eppendorf tubes and centrifuged to pellet the cells at 400 x g for 5 minutes at 20°C. The supernatants were collected in clean tubes and stored at -20°C until analysis. The percentage composition of the oxysterols mixture used was: 7α-HC (5%), 7β-HC (10%), 5α,6α-EC (20%), 5β,6β-EC (20%), triol (9%), 7-KC (35%), and 25-HC (1%).

2.4 Measurement of cytokine/chemokines production

Cytokines were preliminarily measured in cell culture supernatants (400 µl) of two samples, control and treated with the oxysterols mixture 20 µM in ethanol, by a semi-quantitative method to simultaneously detect the relative levels of 36 different cytokines and chemokines using the *Human Cytokine Array Panel A* (R&D systems, Abingdon, UK) following the manufacturer instructions The membranes were developed using ImageQuant LAS 4000 mini. Signal intensities of each membrane array were analyzed using the ImageQuant software (Molecular Dynamics, Amersham Pharmacia Biotech). Three cytokines were selected for quantitative analysis by ELISA on the basis of the initial screening process described earlier. Levels of human cytokines IL-1β, MIF and RANTES were quantified using appropriate kits (*"Human IL-1β/IL-1F2 DuoSet*", "*Human MIF DuoSet*" and "*Human CCL5/RANTES DuoSet"* R&D systems, Abingdon, UK), following the manufacturer's instructions.

2.5 Measurement of intracellular ROS production

Intracellular ROS production was measured using the probe DCFH-DA.

158 PBMCs ($1x10^6$ cells/ml) were seeded in 24 well plate in complete RPMI. In the first set of
159 experiments cells were incubated for 30 min with DCFH-DA 10 μM in the dark at 37°C. DCFH-
160 DA was then removed, and cells were washed with PBS and incubated with the oxysterols mixture
161 20 μM in ethanol added in fresh medium. DCFH-DA loaded cells were immediately placed in a
162 plate reader (Plate reader, Infinite 200, GENios TECAN) setting the excitation filter at 485 nm and
163 the emission filter at 530 nm, with temperature maintained at 37°C. ROS production was monitored
164 by reading the fluorescence emitted taking readings at intervals of 30 min for 3 h. In the second set
165 of experiments cells were pre-treated or not with pure phenolic compounds, HT, TYR and HVA
166 (0.25, 0.5, 1 μM) and incubated for 30 min at 37°C in a 5% CO_2 atmosphere. Cells were then
167 washed, treated with DCFH-DA 10 μM and incubated for 30 min in the dark at 37°C. DCFH-DA
168 was then removed, cells were washed and incubated with the oxysterols mixture 20 μM added in
169 fresh medium for 2 h. The fluorescence emitted from the cells was measured with the same method
170 for 2 h.
171
172 *2.6. Isolation of proteins and western blot analysis of JNK 1/2 and p38*
173 PBMCs ($5x10^6$ cells/ml) were seeded in 6 well plate in complete RPMI, pre-treated or not with HT,
174 TYR and HVA (0.25, 0.5, 1 μM) and incubated for 30 min at 37°C in a 5% CO_2 atmosphere. The
175 oxysterols mixture 20 μM in ethanol was then added in the medium and cells incubated for 3 h.
176 Well contents were collected and centrifuged at 400 x g for 5 min at 4°C. Supernatants were
177 discarded and cells washed/centrifuged twice with cold PBS. Finally the pellets were suspended
178 with 150 μL of complete lysis buffer (Cell Lytic M, Sigma) with protease and phosphatase
179 inhibitors (COmplete Ultra tablets and Mini, PhosphoSTOP, Roche) and incubated on ice for 30
180 min (by vortexing every 10 min). At the end, lysates were centrifuged at 15000 x g for 5 min at 4°C
181 to remove any particles or cell debris and the supernatants were collected for protein analysis. The
182 protein concentration was determined by the Bradford protein assay [39], and 20 ug of protein
183 sample were subjected to SDS-page and western immunoblotting as previously described [40]. The

antibodies used were anti-pp38 (1:1000 dilution), anti-p38 (1/1000 dilution), anti-pJNK 1/2 (1:200 dilution), anti-JNK 1/2 (1:200 dilution) and goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000 dilution). The blots were exposed to Hyperfilm-ECL and developed with the ChemiDoc XRS Imager (BioRad). Protein bands were quantified using Image J software.

2.7 Statistical analysis

The statistical significance of results was evaluated by one-way ANOVA followed by Bonferroni's multiple comparison post-test using GraphPad Prism V5 (GraphPad Software, San Diego, CA, USA). P values of <0.05 were considered statistically significant.

3. Results

3.1 Effect of olive oil phenolics on oxysterol-induced cytokine secretion

After an initial screening with 36 cytokines with a human cytokine proteome profiler array, 3 cytokines that were most significantly modulated by oxysterols were selected for further study. The levels of 3 selected cytokines (IL-1β, MIF and RANTES) were further subjected to quantitative analysis, which indicated that a 24h exposure of PBMCs to oxysterols (20 μM) resulted in a significant increase in the secretion of the pro-inflammatory cytokines/chemokines, IL-1β (Fig.1A), MIF (Fig.1B) and RANTES (Fig.1C) in PBMCs. Pre-treatment with HT, TYR and HVA (0.25, 0.5, 1 μM), significantly reduced the secretion of pro-inflammatory cytokines/chemokines induced by the oxysterols.

3.2 Olive oil phenolics inhibit oxysterol-induced intracellular ROS production

Oxysterols (20 μM) also significantly increased intracellular ROS production over a 180 min timeframe (Fig 2). Pre-treatment of PBMCs with HT, TYR and HVA (0.25, 0.5, 1 μM) for 30 min prior to the addition of oxysterols led to a reduced intracellular ROS production at concentrations of 0.5 and 1.0 μM (Fig.3), with HT and TYR significantly more active than HVA.

3.3 Effect of olive oil phenolics on redox-sensitive pathways involved in oxysterol-induced cytokine production

The oxysterol mixture induced an increase in the level of the phosphorylated forms of JNK 1/2 (p-JNK 1/2) in PBMCs following 3 h of exposure to PBMCs. All polyphenols were shown to significantly attenuate these increases with the exception of HVA, at 0.5 μM. (Fig.4). Similarly, oxysterols also induced the phosphorylation of p38 in PBMCs, with all phenolic compounds counteracting these effects at the highest concentration and HT also active at 0.25 μM.

4. Discussion

Cholesterol oxidation products, termed oxysterols, may either originate endogenously, through enzymatic or non-enzymatic reactions, or may derive from the diet. As regards exogenous sources of oxysterols, foods containing cholesterol are susceptible to oxidation: oxidative reactions occur during food processing, mainly on exposure to heat treatment and during long-term storage [41]. They are involved in physiological processes such as the regulation of cholesterol homeostasis, but it is well established that oxysterols have mostly detrimental biological activities. They provoke an imbalance of the ratio between oxidative and reductive biochemical reactions (oxidative stress) which acts on all organism levels, from cell signalling to disease expression through up-regulation of inflammation, apoptosis and fibrosis [7, 42].

Pathological accumulation of oxysterols may contribute in fact to the onset and especially to the development of major chronic diseases in which inflammation, but also oxidative damage and to a certain extent cell death, are hallmarks and primary mechanisms of progression. Indeed, certain oxysterols exercise strong pro-oxidant and pro-inflammatory effects at concentrations detectable in the lesions typical of atherosclerosis, neurodegenerative diseases, age-related macular degeneration, and other pathological conditions characterized by altered cholesterol uptake and/or metabolism [6]. Several studies in animals and humans have shown that dietary oxysterols, after digestion can be absorbed from the gut and transported into the circulation within chylomicrons and other

237 lipoproteins [43, 44]. Furthermore, the presence of oxysterols in plasma can derive from the
238 oxidation of endogenous cholesterol through enzymatic or spontaneous reactions [7].
239 Oxysterols have been found at increased levels in the plasma of hypercholesterolemic subjects and
240 have been linked with the atherosclerotic process [45].
241 This study focused on the protective effect of pure olive oil phenolics, which can be found in the
242 blood stream after absorption (HT, TYR, and HVA) against the pro-oxidant and pro-inflammatory
243 activity of oxysterols in immune blood cells.
244 The phenolic compounds used in our study are bioavailable [46-49], as shown in numerous studies,
245 and the concentrations used in this study are physiologically relevant (0.25 - 0.5 - 1 μM) [50].
246 PBMCs separated from whole blood of healthy volunteers were used for this study; these are
247 composed by lymphocytes, monocytes and macrophages, critical components in the immune system
248 to fight inflammation and source of pro-inflammatory molecules.
249 PBMCs cells were treated with an oxysterols mixture composed by the most widely represented
250 oxysterols in plasma of hypercholesterolemic subjects: 7α-HC, 7β-HC, 7-KC, 5α,6α-EC, 5β,6β-EC,
251 triol and 25-HC [38] at pathologically relevant concentration (20 μM). In fact, oxysterols in human
252 plasma or serum may vary from about 1 μM (0.05% of total cholesterol) in healthy subjects to 20-
253 30 μM (0.5-0.75% of total cholesterol) in diseased individuals, but much higher concentrations of
254 plasma oxysterols have also been reported [4, 45, 51].
255 The first intent was to examine the ability of PBMCs to produce cytokines and chemokines in the
256 presence of oxysterols, in particular, IL-1β, MIF and RANTES, usually involved in pro-
257 inflammatory processes; the oxysterols mixture was able to significantly increase the secretion of
258 all cytokines/chemokines analysed. Since it has been suggested that oxysterols may increase the
259 levels of cytokines by modulating redox-sensitive pathways [52], the oxidative status of PBMCs
260 treated with the oxysterols mixture was then measured. An early increase (after 15 min) of
261 intracellular ROS production was observed in PBMCs challenged with oxysterols compared to
262 control. At moderate concentrations, ROS may act as second messengers in signal transduction, by

263 modulating redox-sensitive MAPK; these kinases have been already reported to be activated by
264 various stress stimuli, including treatments with different oxysterols, 7-KC and 25-HC [52, 53] and
265 they have been also implicated in oxysterol-induced cytokine secretion and apoptosis [6, 52, 54].
266 In the experimental conditions of this research, the oxysterols mixture induced a significant increase
267 of both JNK and p38 phosphorylation suggesting their involvement in cytokine secretion.
268 These results are in accordance with other experimental studies; for example, in human monocytic
269 cells, 7β-HC and 25-HC, but also 7-KC to a lesser extent, are potent in vitro inducers of IL-1β, IL-
270 8, TNF-α, and MIP-1β, as well as of other inflammatory molecules [55]. The same study
271 demonstrated that IL-8 secretion was associated with activation of the ERK 1/2 signaling pathway
272 [55]. The oxysterols 7-KC and 25-HC have also been observed to enhance IL-1β, IL-6, IL-8, and
273 TNF-α mRNA and secretion levels, in a dose-dependent manner, although to different extents.
274 These effects were associated with increased ROS production, and a net phosphorylation of MAPK
275 (ERK 1/2, JNK, p38) and NF-*k*B activation also occurred [52].
276 Up-regulation of IL-1β is another important event, because this cytokine increases the surface
277 expression of endothelial adhesion molecules, by facilitating inflammatory cells attachment to the
278 artery endothelium. Expression and synthesis of IL-1β were found to be stimulated by 25-HC in
279 human macrophages, through the involvement of the liver X receptor, as well as, but less strongly,
280 by 27-hydroxycholesterol (27-HC) [56]. IL-1β secretion was also markedly induced by 7β-HC, 7-
281 KC, and 7α-HC in human promonocytic cells U937 and U4 [57, 58] and in human umbilical vein
282 endothelial cells (HUVECs) [59]. Production of the pro-inflammatory cytokines TNF-α and IL-1β
283 is also induced by 25-HC in adherent human peripheral blood mononuclear leukocytes, through
284 phosphorylation of p38 MAPK [60]. In the literature there is not any specific correlation between
285 oxysterols and RANTES and MIF secretion; but the increased RANTES and MIF expressions have
286 been associated with a wide range of inflammatory disorders and pathologies, such as inflammatory
287 bowel disease and atherosclerosis where oxysterols exert a pivotal role. RANTES and MIF are
288 cytokines with chemokine-like function and critical mediators of the host immune and

289 inflammatory response; they are thought to act by recruiting and promoting leukocytes infiltration
290 to sites of inflammation [61, 62].
291 In this study it was demonstrated that all phenolic compounds HT, TYR and HVA at all tested
292 concentrations, were able to inhibit oxysterol-induced pro-inflammatory cytokines production *in ex-*
293 *vivo* immune cells. According to the hypotesis that pro-inflammatory cytokines release may be
294 induced by changes in intracellular redox status, it was observed that, in human PBMCs treated
295 with the oxysterols mixture, simple phenols were able to inhibit ROS production significantly from
296 the concentration of 0.5 μM as well as to suppress redox-based MAPK phosphorylation (JNK, p38).
297 In the literature there are several studies that confirmed the anti-inflammatory activity of olive oil
298 phenolics in different districts such as in blood cells. Differential anti-inflammatory effects of
299 phenolic compounds from extra virgin olive oil were identified in human whole blood cultures: OL-
300 glycoside and CA decreased the concentration of IL-1β and kaempferol decreased the concentration
301 of PGE2 induced by LPS [15].
302 A recent study reported similar results in macrophages where oil phenolic extract exerted their
303 protective effects against LPS-induced oxidative stress and inflammatory responses [24, 25]. The
304 olive oil phenolic extract significantly decreased NO and ROS production and in addition
305 significant down regulated iNOS, COX-2, reduced MAPK (JNK, p38) phosphorylation and
306 prevented the nuclear NF-*k*B traslocation.
307 HT inhibits iNOS and COX-2 expression in LPS-stimulated J774 cells at the transcriptional level
308 by preventing the activation of NF-*k*B, signal transducer and activator of transcription 1 (STAT-1α)
309 and interferon regulatory factor 1 (IRF-1) [16] and TYR prevented RAW 264.7 macrophages
310 activation induced by gliadin and INFγ [63].
311 A recent study conducted by Palozza et al. [52] investigated the ability of lycopene, a strong
312 antioxidant compound present in tomato, to prevent oxysterols induced pro-inflammatory cytokine
313 cascade in human macrophages. Lycopene prevented oxysterol-induced increase in pro-
314 inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α) secretion and expression, such an effect was

315 accompanied by an inhibition of oxysterols-induced ROS production and MAPK activation (ERK
316 1/2, JNK and p38). In our study, Olive oil phenolics showed similar capabilities against the harmful
317 effects of the oxysterols mixture *in ex-vivo* blood cells.
318 Further studies are needed to clarify the mechanism by which the phenolic compounds exert their
319 protective action; however, data obtained in these experimental systems, suggested that these
320 compounds act primarily by counteracting the initial stages of the pro-oxidant and pro-
321 inflammatory effects of oxysterols, inhibiting the formation of ROS and then all subsequent
322 cascading effects.

Author contributions: *G.S., M.D., and G.C. designed the research; G.S. conducted the research; G.S. analysed the data; G.S. wrote the manuscript; G.C., M.D. and J.P.E.S. revised the manuscript. G.S. and G.C. had primary responsibility for the final content. All authors read and approved the final manuscript.*

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Figure legends:

Figure 1: Effects of HT, TYR and HVA (0.25-0.5-1 μM) on the pro-inflammatory cytokines IL-1β **(A)**, MIF **(B)** and RANTES **(C)** secretion in PBMCs treated with the oxysterols mixture 20μM for 24h. Each column represents the mean ± SD of six independent experiments.

*** = $p<0.001$ vs Ctrl; a = $p < 0.001$, b = $p< 0.01$, c = $p< 0.05$ vs oxy

Control value: IL-1β = 91 pg/ml, MIF = 770 pg/ml, RANTES = 2800 pg/ml

Figure 2: Intracellular ROS production (expressed as fold increase) in PBMCs treated with the oxysterols mixture 20 μM for different incubation times (15-180 min) using the fluorescence probe DFC-DA 10 μM for 30 minutes. Each column represents the mean ± SD of six independent experiments. *** = $p<0.001$ vs ctrl

Figure 3: Effect of HT, TYR and HVA (0.25- 0.5- 1 μM) on intracellular ROS production in PBMCs treated with the oxysterols mixture 20 μM for 2 h. Each column represents the mean ± SD of six independent experiments.

*** = $p<0.001$ vs Ctrl; a = $p<0.001$, b = $p<0.01$, c = $p<0.05$ vs Oxy

Figure 4: Modulation of JNK 1/2 in PBMCs pre-treated or not for 30 min with HT, TYR and HVA (0.25-1 μM) and incubated with the oxysterols mixture 20 μM for 3h. Each column represents the mean ± SD of three independent experiments. ***= $p<0.001$ vs Ctrl; a = $p<0.001$ vs Oxy

Figure 5: Modulation of p38 in PBMCs pre-treated or not for 30 min with HT, TYR and HVA (0.25-1 μM) and incubated with the oxysterols mixture 20 μM for 3h. Each column represents the mean ± SD of three independent experiments.

***= $p<0.001$ vs Ctrl; a = $p<0.001$, c = $p<0.05$ vs Oxy

Figure 1.

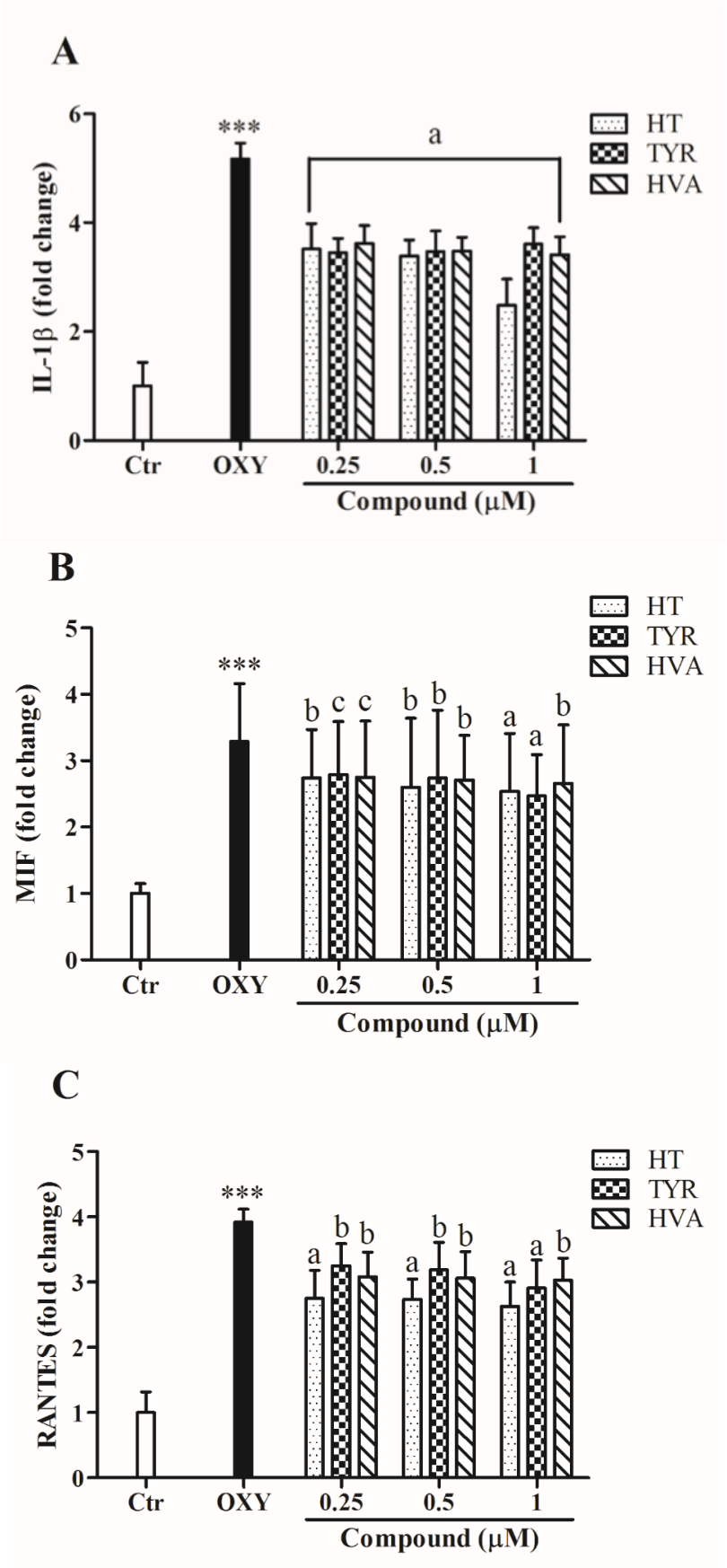

A
IL-1β (fold change)
***
a
HT
TYR
HVA
Ctr
OXY
0.25
0.5
1
Compound (μM)
B
MIF (fold change)
***
b c c b b b a a b
HT
TYR
HVA
Ctr
OXY
0.25
0.5
1
Compound (μM)
C
RANTES (fold change)
***
a b b a b b a a b
HT
TYR
HVA
Ctr
OXY
0.25
0.5
1
Compound (μM)

Figure 2.

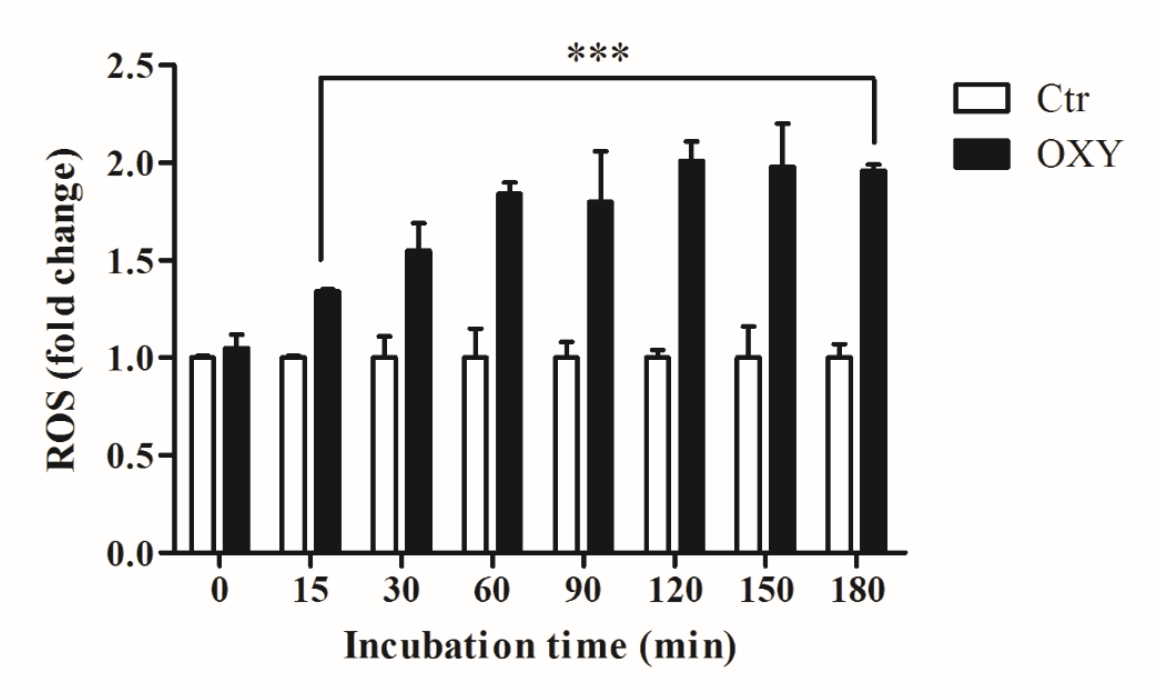

***
Ctr
OXY
ROS (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
0
15
30
60
90
120
150
180
Incubation time (min)

Figure 3.

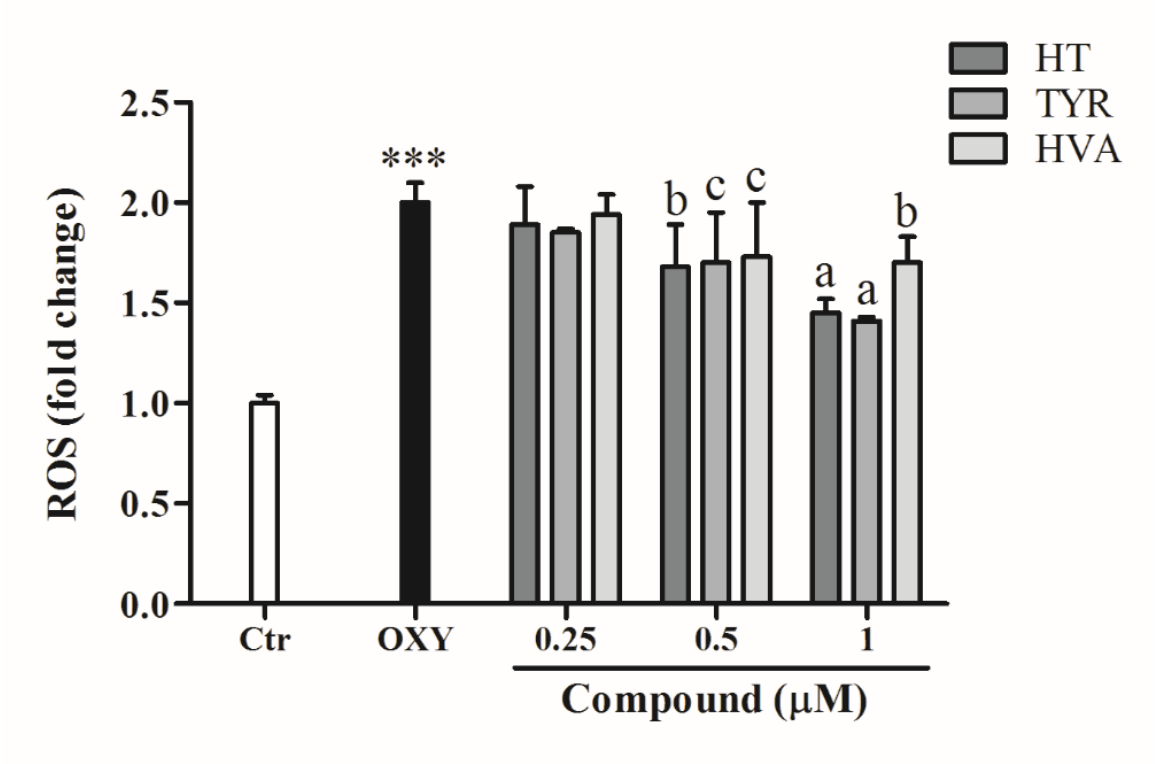

HT
TYR
HVA
ROS (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
***
b
c
c
a
a
b
Ctr
OXY
0.25
0.5
1
Compound (μM)

Figure 4.

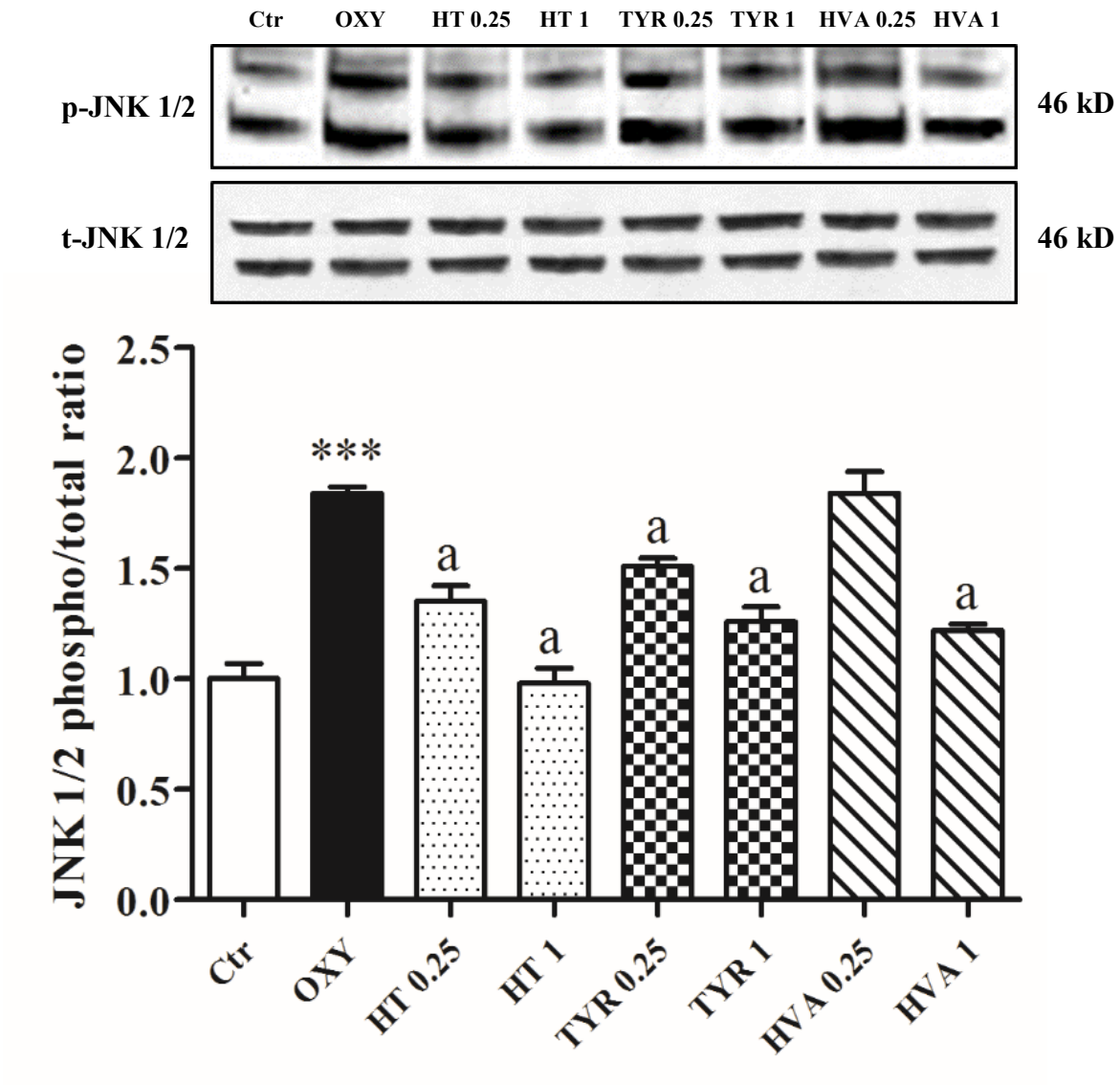
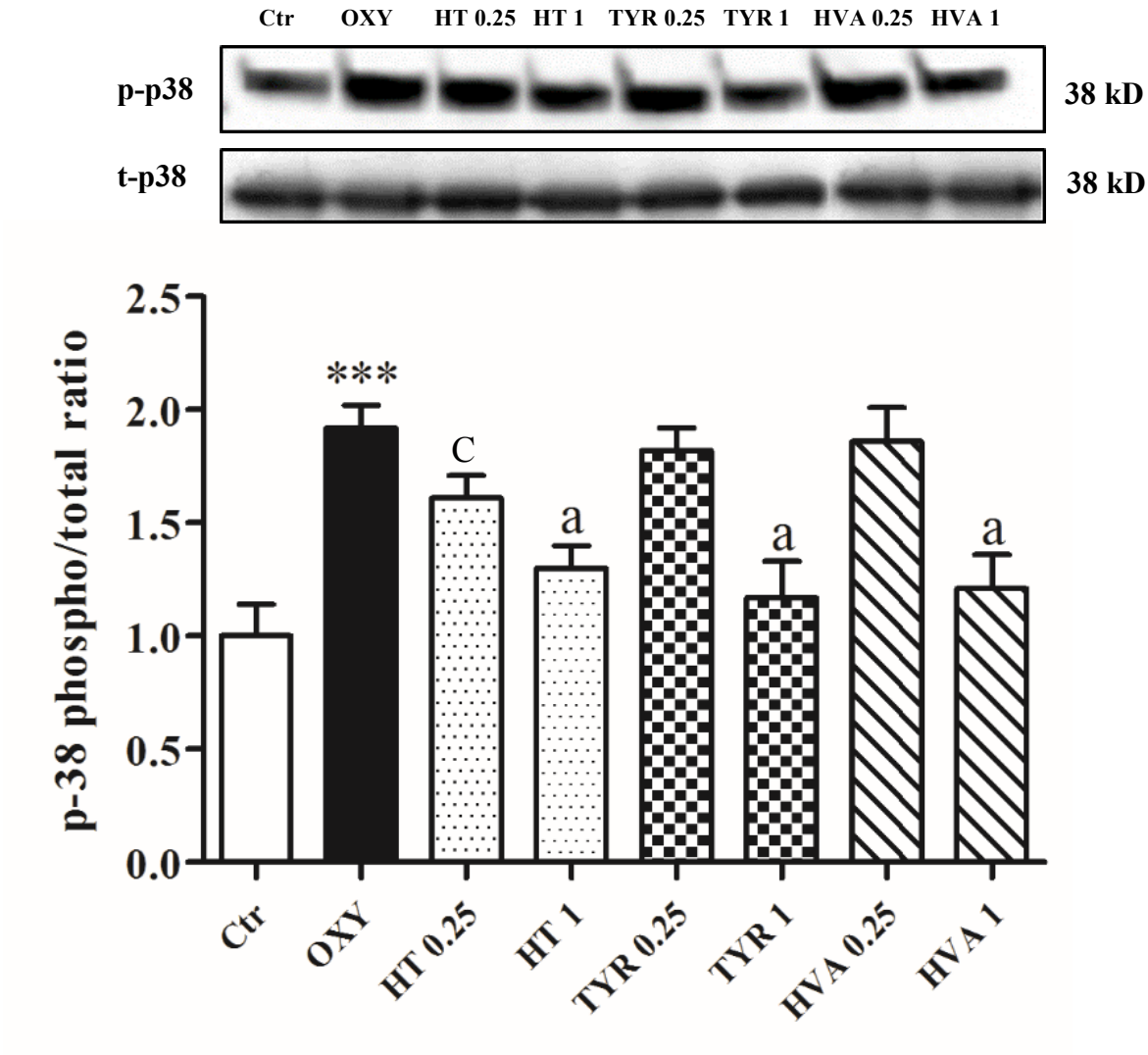

Ctr
OXY
HT 0.25
HT 1
TYR 0.25
TYR 1
HVA 0.25
HVA 1
p-JNK 1/2
46 kD
t-JNK 1/2
46 kD
JNK 1/2 phospho/total ratio
2.5
2.0
1.5
1.0
0.5
0.0
***
a
a
a
a
a
Ctr
OXY
HT 0.25
HT 1
TYR 0.25
TYR 1
HVA 0.25
HVA 1

Figure 5.


Ctr
OXY
HT 0.25
HT 1
TYR 0.25
TYR 1
HVA 0.25
HVA 1
p-p38
38 kD
t-p38
38 kD
p-38 phospho/total ratio
2.5
2.0
1.5
1.0
0.5
0.0
***
C
a
a
a
Ctr
OXY
HT 0.25
HT 1
TYR 0.25
TYR 1
HVA 0.25
HVA 1